Material & Methods

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# Presentation of the technique [This part will be in the Results]

## Overview -> WRITE

The Magnetic Pincher setup consists of an inverted microscope used in bright field illumination, with a system mounted on the microscope stage to generate a uniform magnetic field at the location of the imaged sample. This system can be an array of permanent magnets or a pair of electromagnetic coils. The protocol, whose main steps are summarized on Fig. 1, is based on 4.5 µm superparamagnetic microbeads, the M-450 Dynabeads. Cells are incubated with these beads until a significant uptake is reached. Then, cells are transferred in the experimental chamber, together with additional beads. The chamber, placed on the microscope, is exposed to a controlled uniform magnetic field. The beads magnetize and attract each other at short range, forming pairs or chains of beads. This self-organization generates simultaneously a large number of pinching events in the chamber (Fig. 2A, 2D), meaning a situation where a bead inside the cell forms a pair with another outside the cell, which pinches the cell cortex. Such beads can then be filmed under bright field illumination, and the images analyzed with a simple algorithm tracking the beads positions (Fig. 2B). This way we compute the cortical thickness and its evolution in time (Fig. 2C) with a high spatial (≈ 30 nm) and temporal resolution (up to 100 Hz).

## Cell lines

## Micropatterning

3T3 fibroblasts are adhering cells that spread a lot when cultured on a 2D substrate. When they have this shape, it is difficult to use the Magnetic Pincher because of what we nicknamed the “tent-pole effect”: the inner bead is larger than the typical height of a spread fibroblast. The bead will be brought toward the center of the cell where it acts as a “tent-pole”. Because of the curvature of the cell surface, it is very hard in these conditions to form a pair with another bead outside; and if it happens, the beads are usually in different planes, thus impossible to image.

On the other hand, 3T3 cells do not survive for a very long time when they cannot adhere to a substrate. This is why we used fibronectin micropatterns to limit their spreading without preventing them from adhering. More precisely, we developed a protocol to make fluorodish-like chambers, where the glass bottom is passivated with PLL-Peg, and micropatterned with fibronectin discs.

Since the average diameter of 3T3 cells in suspension is roughly 18 µm, they form a quasi-hemispheric shape when adhering on discs that have a diameter close to this value. Thus, we picked 20 µm as our standard pattern size.

## Magnetic Field

This technique requires the generation of a uniform magnetic field over the experimental chamber. When exposed to an external magnetic field, superparamagnetic beads will become magnetic dipoles, with a magnetic moment that is a function of the applied field. Because the gradient of the external field is negligible, there is no long-range magnetic force and the beads do not drift. Instead, the dipolar force generated by each bead causes them to attract each other. This leads to a self-organization of the beads present in the experimental chamber, forming pairs or chains aligned with the magnetic field (Fig. 5A, 4B). We insist here on the importance of the field uniformity: too strong a gradient of magnetic field would cause superparamagnetic beads to drift toward the higher field regions.

## Magnetic Beads

## Magnetic Pincher & Image analysis

# Cell Culture

Except when otherwise, cells assumed to be 3T3 fibroblasts, purchased from LGC standards.

## 3T3 ATCC-2023 fibroblasts

Purchased from LGC standards in January 2023

Amplified and frozen at low passage numbers

Used as a base for the creation of a stable line expressing LifeAct-EGFP

Culture medium: Dulbecco’s Modified Eagle Medium (DMEM) with GlutaMAX (61965026, Thermofischer) and supplemented with fetal bovine serum (FBS — 10% of final volume, S1810-500, Biowest, France), penicillin-streptomycin (PS — 1% of final volume, 15070063, Thermo Fisher, USA).

Incubated at 37°C in 5% CO2 atmosphere (Memmert, ICO50)

## 3T3 αSFL fibroblasts

Reference to Sergio, Leanne & Alba’s paper // Cells were cultured by me in our lab, by making complete medium similar to their using our DMEM but their FBS

Cell line creation (in their lab)

Cell culture methods (in our lab)

Reagents

## HOX B8 macrophages

Reference to Perrine & Renaud’s paper // Cells were only used for a short duration and cultured by RP

### REWRITE / Murine HoxB8-progenitors culture and HoxB8-macrophages differentiation

Myeloid progenitors were isolated from the bone marrow of a mouse and immortalized by transduction with a retrovirus allowing conditional expression of the HoxB8 homeobox gene, as previously described (Verdys et al., 2024 ; Accarias et al., 2020). HoxB8 progenitors were then passaged every 2 days in myeloid medium [complete RPMI 1640 medium, supplemented with 20 ng/mL mouse GM-CSF (Miltenyi #130-095-739) and 0.5 μM β-estradiol (Sigma-Aldrich #E2758)]. To differentiate them in macrophages, progenitors were washed twice in PBS to remove estradiol from the medium, and 500,000 cells were plated in 6-well-plate in complete medium [complete RPMI 1640 medium (Gibco)] containing 20 ng/mL of mM-CSF.

### REWRITE / Transfection, transduction, and sequence analysis for the creation of single and triple-knockout in murine HoxB8 progenitors

SgRNAs used for knocking out ERM proteins were EZR, RDX and MSN.

For the triple ERM-knockout, all three sgRNAs were cloned on a same plasmid by golden gate assembly (Kabadi et al., 2014). All intermediary plasmids containing respective sgRNA were then cloned into the final pLV hUbC-Cas9-T2A-GFP plasmid (Addgene #53190), where GFP was replaced by puromycin resistance.

Then, HoxB8 progenitors were knocked-out for ezrin, radixin and moesin using CRISPR Cas9. Briefly, HEK293T cells were co-transfected with pMDL (Addgene #12251), pREV (Addgene #12253), pVSV-G (Addgene #12259) and specific sgRNA-cloned plasmids using Lipofectamine 3000 and OptiMEM according to manufacturer’s guidelines to generate lentiviral particles for transduction into Cas9-expressing HoxB8 progenitors. Then, 2×105 HoxB8 progenitors were transduced with viral particles and Lentiblast Premium (OZ Biosciences). After 24h, transduced cells were selected with 10 µg/mL of puromycin (Invivogen) for 2 days and validated by immunoblotting. A clonal amplification was performed by single cell sorting and clones were screened for ezrin, radixin and moesin absence of expression by flow cytometry. Immunoblotting with respective ERM antibodies confirmed the depletion of the three ERM proteins in three independent clones (tKO#1, tKO#2, tKO#3).

### QUESTIONS FOR PV / Cell preparation methods (in our lab):

* How many days after differenciation?
* Plating concentration and specific handling

## Cell lines conservation

3T3 cells (αSFL and ATCC-2023) were systematically amplified and frozen at low passage numbers. Cells were frozen in 1mL aliquots of roughly 2 millions cells/mL. To do so cells were detached using TrypLE (12605036, Thermo Fisher, USA), centrifuged in culture medium … of a mix of fetal calf serum (50%), culture medium (45%) and DMSO (5%). They were conserved at -150°C and thawed when needed. The total passage numbers was always kept below 20, and rarely went above 10 throughout this work.

# LifeAct-EGFP Transduction

A stable cell line of 3T3 ATCC-2023 fibroblasts expressing LifeAct-EGFP was produced by lentiviral transduction followed by FACS sorting.

I prepared a mix of 200 µL of OptiMEM (Thermofischer), 4 µL of Lipofectamin 2000 (Thermofischer) and 2 µg of DNA, which include:

- 0.3 µg of pMD2.G — envelope protein (gift from Francois-Xavier Gobert, Institut Curie)

- 0.8 µg of PsPax2 — reverse transcriptase, capside, integrase (gift from Francois-Xavier Gobert, Institut Curie)

- 0.9 µg of the plasmid of interest — pLenti Lifeact-EGFP BlastR (Addgene Plasmid #84383)

I plated 1.6 million HEK-293FT cells (gift from the lab of Nicolas Manel) in 2mL of DMEM (ref 61965059, Thermofischer) and supplemented with the mix. 4 hours later I replaced the medium with 3mL of the 3T3 culture medium.

The next day I harvested the viruses by aspiring the medium with a syringe and filtered it with a 0.45 µm membrane. I added 1 mL of virus solution in three wells of a 6-wells plate containing cultures of 3T3 ATCC-2023 fibroblasts at different concentrations (around 10, 25 and 50% confluency).

The third day I washed the 3T3 cells twice with PBS and renewed the medium. On the fourth day I assessed in which of the three wells the cells had the most fluorescence signal while retaining a normal phenotype. I amplified these cells and proceeded to a FACS sorting to keep only cells within a narrow window of LifeAct-EGFP expression. These cells were amplified and frozen. The resulting stable cell line was called 3T3 ATCC LaGFP 02-01.

# Micro-patterned Chambers

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| *# This could be placed here or before, in the presentation of the technique in the results section.*  3T3 fibroblasts are adhering cells that spread a lot when cultured on a 2D substrate. When they have this shape, it is difficult to use the Magnetic Pincher because of what we nicknamed the “tent-pole effect”: the inner bead is larger than the typical height of a spread fibroblast. The bead will be brought toward the center of the cell where it acts as a “tent-pole”. Because of the curvature of the cell surface, it is very hard in these conditions to form a pair with another bead outside; and if it happens, the beads are usually in different planes, thus impossible to image.  On the other hand, 3T3 cells do not survive for a very long time when they cannot adhere to a substrate. This is why we used fibronectin micropatterns to limit their spreading without preventing them from adhering. More precisely, we developed a protocol to make fluorodish-like chambers, where the glass bottom is passivated with PLL-Peg, and micropatterned with fibronectin discs.  Since the average diameter of 3T3 cells in suspension is roughly 18 µm, they form a quasi-hemispheric shape when adhering on discs that have a diameter close to this value. Thus, we picked 20 µm as our standard pattern size.  *# It’d be nice to have a figure to illustrate the situation of beads in/out the cell on different patterns* |

**Photomask design.** We designed the masks geometry with the software CleWin5. The masks were purchased from JD-Photodata (UK). We used masks made of quartz, because glass absorbs the deep-UV used for the patterning.

**Petri dish preparation.** We use 35 mm petri dishes with a 20 mm hole cut in the plastic bottom. Such dishes can either be bought or fabricated. We used a laser cutter (Epilog Laser, USA), which can cut dishes by batches with good precision on the centering.

**Coverslip passivation with PLL-Peg**. Round glass coverslips (diameter 25 mm, thickness #1) are cleaned with ethanol and exposed to plasma for 2 minutes. Then they are placed over 50 µL droplets of a 0.1 mg/mL PLL-Peg solution [PLL(20)-g[3.5]-PEG(2) from SuSoS, Switerland, diluted in Hepes 10 mM pH 7.4, and filtered with a 0.45 µm membrane]; the coverslips are incubated with the solution for 40 minutes. They are then removed, rinsed with milliQ water and gently dried with a tissue (without contact with the coated surface).

**Micropatterning with deep-UV.** The mask was cleaned with isopropanol, then the metallic side was exposed to deep-UV for 10 minutes. We used a UV lamp with wavelength λ = 254 nm and power P = 7 mW/m² (UVO Cleaner, Jelight) placed under a fume hood because of ozone emissions. Then, for each coated coverslip, a drop of 11 µL of milliQ water was placed on the metallic surface of the mask. Coverslips were gently placed on top of these droplets, coated side down, so as not to retain any air bubble between the glass and the mask surface. Then remove the excess of water by blowing compressed air from the top: each coverslip should be stuck to the mask by a very thin water film. The mask was exposed again to deep-UV-light for 10 minutes, this time with the non-metallic face toward the UV source (so that the light properly go through the mask before reaching the coverslips). Finally, the mask was covered with a puddle of milliQ water to allow the coverslips to detach. They were removed and dried.

**Experimental chamber manufacturing.** We used non-toxic silicon glue (Silicone SA 500, Zolux, France), to attach the patterned coverslip to the cut petri dish. Using a syringe, the glue was applied in a thin line around the hole, on the bottom side of the dish. A PLL-Peg patterned coverslip is then gently pressed onto the circle of glue. The glue was left to dry for at least 4 hours.

**Fibronectin addition.** We used either a solution of fibronectin (#F1141, Sigma-Aldrich) [10 µg/mL fibronectin in NaHCO3 buffer pH 8.3, filtered with a 0.2 µm membrane] or a mix of fibronectin and Alexa Fluor™-conjugated fibrinogen (typically Alexa Fluor™ 647, #F35200, ThermoFischer) [same recipe as before plus 4µg/mL fibrinogen], which allow a precise visualization of the patterns. Patterned dishes were incubated for 30 minutes with 125 µL of solution (using parafilm discs to spread the solution on the glass surface). The dishes were then rinsed twice with PBS.

# Magnetic Beads properties

## Choice of the magnetic bead type

The properties of the superparamagnetic beads are crucial to the feasibility and the precision of the Magnetic Pincher technique. The size of the beads in particular is key: they need to be big enough to generate a sufficient dipolar force and for their position to be accurately determined with bright field microscopy. We have identified the M-450 Epoxy Dynabeads (Dynal, Thermo Fisher, USA) as the best choice since they ally many important features:

1. Beads from the Dynabead line are very monodisperse in diameter. A given batch of M-450 Dynabeads typically has an average diameter close to 4.5 µm with a standard deviation of less than 25 nm. Because the average diameter varies from batch to batch,
2. M-450 possess one of the highest density of magnetic particles [12] and the largest size of all Dynabeads. Thus they can acquire high magnetic moment magnitudes, which result in a large range of pinching forces: from 1 pN to more than 1 nN. Moreover, like other Dynabeads, they have a very small residual magnetization, which ensures that they stop attracting when the field is brought back to zero.
3. Optical properties: when observed under transmitted bright light illumination at high magnification, an intense light spot forms below the bead center (Fig. 5C). By computing the center of mass of this light spot, using the light intensity (pixel value) as a weight we can localize the center of the beads with a resolution that overcomes the diffraction limit.
4. They are relatively easy to take up for cells. This depend on the cell type and can be tuned by coating the beads with diverse molecules, but every cell type considered for this experiment so far have proved able to take up M-450 beads.

This technique have also been performed successfully using M-270 beads, which are smaller (typical diameter: 2.7 µm) and magnetize less strongly. The method did not work when attempted with MyOne beads (typical diameter: 1 µm). The M-450 beads are nonetheless the best choice in terms of robustness and precision.

## Tracking in 3D

When observed under transmitted bright light illumination at high magnification, an intense light spot forms below the bead center (Fig. 5C). Computing the center of mass of this light spot, using the light intensity (pixel value) as a weight allows the center of the beads to be localized with a resolution that overcomes the diffraction limit: the precision of their localization in the XY place is 2 nm [8, 13]. In practice, this detection of the center in 2D is a very simple ImageJ routine (see Mat. & Meth).

However, beads pinching a cell are not always at the same altitude: the distance along the z-axis often has a significant contribution. It is then necessary to detect the relative positions of the beads in 3D, which is a little bit trickier. To do so, with standard image analysis techniques (no Machine Learning here yet!) our method is based on the generation of a “depthograph” — a typical YZ-profile of the light patterns formed below the beads, just like a kymograph in depth. It serves as a common reference: if for an image of beads, we can find for each beads where they are in the depthograph, we know their relative positions along the z-axis.

To make depthographs, we take Z-stacks of beads (401 images with a 20 nm step, from the equatorial plane downward). Then on each frame we draw a vertical line passing by the center of the bead, and we take the pixel intensity profile along this line. This gives us a 2-dimensional map of the bead depth in the YZ-plane. We repeat this operation for several beads and average them together to obtain our depthograph.

Then, to localize beads in a frame of a time-lapse, we detect their centers in XY using the method described above. For each bead, we proceed as before, drawing a vertical line passing by the center of the bead, and taking the pixel intensity profile along this line. This profile is compared to the depthograph to find the best matching position, which is the line of the depthograph that minimizes the L2-distance to the profile (see mat. & meth). Doing so for each bead of the frame allow us to find their relative positions along z, using the depthograph as a common reference.

In practice, we use this algorithm on the 2 beads pinching the cortex of a cell. In each frame of the timelapse, we determine the center-to-center distances along the 3 axis: dx, dy and dz. Finally, the 3D-distance is:

We estimated the error on dz to be of the order of 50nm and the error on D3 to be 10nm. MORE DETAILS.

## Magnetization, magnetic moment and magnetic force

When using superparamagnetic Dynabeads, it is possible to determine the attractive force a bead applies on another nearby bead as a function of the external magnetic field and the bead positions. First, the bead magnetization M (A/m) as a function of the external field B (T or mT) is known from the literature [Ref]. It was fitted by Julien Heuvingh as an empirical function of this form:

The values of parameters a, b, c, α, β, γ are fixed for all bead types. The value of K is adjusted for each bead type and depends of the density of magnetic particle in the beads. The parameter kcorrMag is adjusted for each new batch of beads purchased (see section on the magnetization measurement).

Then, the total magnetic moment of a bead can be expressed as:

with:

Where V and D are the volume and diameter of the bead. Those are measured once for each batch of beads purchased and treated as constant for all beads from this batch.

The magnitude of the attractive force exerted by a bead on another is noted F and expressed as such:

Where m1 and m2 are the two beads’ magnetic moments, d is the center-to-center distance, α is the angle between the direction of the magnetic field and the center-to-center direction, and µ0 is the vacuum magnetic permeability (Fig X). The factor expresses that this force is attractive of maximum magnitude when α = 0 (beads aligned with the field), and decreases as α increases, to become repulsive when α > 55°. Note also that F is proportional to d-4, meaning that the attraction decrease quickly as the distance increases. For instance, if F(d = D) = F0 then F(d = 2.D) = F0/16, and F(d = 3.D) = F0/81.

Typical curves for M(B) and F(B) are shown on Fig X. The typical magnetic field range used in our experiments is 1 to 55mT. Note that the attractive force varies significantly in this interval, but grows slower for higher magnetic fields. This is due to the saturation of M(B), typical of paramagnetic materials. This shows that it would be unproductive to use the Magnetic Pincher with much higher fields: the increase in force would be lower and lower.

## Correction of the magnetic force in a chain of beads

When computing the force applied on the cell cortex by beads in the context of the Magnetic Pincher, the situation is usually a bit more complex, because the beads will tend to attract all their neighbors to form chains aligned with the magnetic field direction. Let’s consider the case where an elastic object is pinched by two arbitrary long chains of beads, represented on Fig. X. The applied force is mostly due to the two beads in contact with the object, but the other beads of the chain also contribute, in two ways.

**Induced magnetic field.** In our systems, the superparamagnetic beads acquire a magnetic moment due to the external magnetic field. This means each beads also generate its own small magnetic field. These fields are mostly much too small to be important, except at very short range. This is the case in chains of beads: each bead’s small magnetic field increase the magnetization of their neighbors. This the magnitude

**Added forces.**

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| BOX — Added forces  Let’s consider the case where an elastic object is pinched by two arbitrary long chains of beads, represented on Fig. X. The applied force is mostly due to the two beads in contact with the object, b-1 and b1. But the other beads of the chain also add a contribution. To clearly identify it, let’s do the balance of forces on the chain (b1, b2, b3, …). The chain is attracted by Fmag the magnetic force exerted by the other chain, and this force is balanced by Fel, the repulsive elastic response of the pinched object. Here, Fmag can be expressed as the sum of all attractive forces applied by a bead of the left-hand chain onto a bead of the right-hand chain:  Where Fi,j is the force applied by the bead i on the bead j. However, each of these terms varies as di,j-4, which means that the contribution of the pairs (b-2, b1) and (b-1, b2) is already about 16 times lower than (b-1, b1). For the next neighbors, it goes does down to 81 times lower, etc. Hence, in all of our calculations involving chains of beads, we neglected the contribution of neighbors beyond the first ones:  C:\Users\JosephVermeil\AppData\Local\Microsoft\Windows\INetCache\Content.Word\ForceMagVoisinsFichier 1@4x.png |

# Magnetic Beads Handling

## Bead size measurement

The distribution of diameters of the M-450 Dynabeads has a very low variance, with the exception of rare and obvious outliers, and varies only slightly from one batch to another. Nonetheless, for each vial of beads newly purchased, it is necessary to measure precisely the average diameter, as it is one of the main sources of uncertainties of the technique. This is done easily using long chains of beads that form under specific conditions.

Briefly, we coated a 35 mm glass-bottom petri dish with 1 % bovine serum albumin (Merck, Germany). We prepared a solution of the beads of interest in PBS (roughly 200’000 beads/mL) and added 2 mL in the dish. We placed this dish in our imaging set-up (transmitted light, objective 100X NA = 1.4) under a uniform magnitude field of magnitude 5 mT. The beads self-organize to form long chains. We took pictures of chains until we had at least 200 beads in total. Using the bead center detection routine detailed above, we determined to distance between all pairs of neighbors in the chains. The average was taken as the central tendency for the bead diameter (in a given batch). Measured values can be found below in table Y.

## Bead magnetization measurement

The formula [Eq. Z] captures well the typical magnetization curve of Dynabeads. However, to account for batch-to-batch variability, we developed a protocol to measure the magnetization of these beads in our lab and adjust the value of accordingly in Eq. Z.

To do so we prepare a small chamber containing a very dilute suspension of beads, which we place in a gradient of magnetic field, using a single electromagnetic coil (Fig. X). Then we film the motion of these beads. In the regime of constant velocity, the force balance projected on the x-axis gives:

The magnetic force experienced by the bead — due to its magnetic moment and the external gradient of field — is balanced by its viscous drag (Fig. X). Being at low Reynolds number, this viscous drag is given by Stokes’ Law : ; where µ is the viscosity of water, R the bead radius and vx the velocity of the bead along the x-axis. On the other hand, once projected, the magnetic force is:

Hence we have the relation:

Using a gauss-meter, we measure the magnetic field & the corresponding gradient produced by the coil according to the current intensity supplied. Then for several values of intensity, we film beads in suspension in water being moved by the magnetic force. As shown on the kymograph on Fig. X, we reach a constant velocity regime and we can measure *vx*. Since R, V and dB/dx are known, we obtain an experimental M(B) curve. By fitting it with Eq. Z, we adjust the parameter and obtain the relation that will characterize this batch of bead magnetization (Fig X). Measured values can be found below in table Y.

Table Y — Measured values for the D and in different batches.

|  |  |  |
| --- | --- | --- |
| **Bead batch** | **D ± std (nm)** |  |
| M450-2020 | 4453 ± | 1.05 |
| M450-2022 | 4503 ± | 1.05 |
| M450-2023 | 4477 ± 18 | 1.023 |
| M450-2025 | 4493 ± 29 | 0.969 |
| M450-Strept | 4506 ± 17 | 1.056 |
| M270-2022 | 2691 ± | 1.05 |

## Bead preparation -> UPDATE

Before using M-450 Dynabeads it is necessary to rinse them from the stock solution medium and coat them with a molecule to tune the ability of cells to ingest them. Our standard coating protocol uses complete medium (with 10% FBS) to moderately increase the uptake rate by cells. Other options are fibronectin coating, to increase bead ingestion, and mPEG coating to decrease it (see Note 2).

Rinsing

1. The commercially available M-450 Dynabeads are conserved in distilled water with a concentration of 4x108 beads/mL. Vortex the stock solution vial to resuspend the beads and pipet 30µL of the solution in an aliquot.
2. Add 1mL of PBS to this aliquot and vortex the mix for 20 seconds.
3. Hold the aliquot vertically above a magnet for 20 seconds. The lower tip of the aliquot should be in contact with the magnet. The beads will sediment rapidly.
4. While holding the aliquot vertically above the magnet, gently remove the supernatant to disturb the bead pellet as little as possible.
5. Repeat steps 2 to 4 twice.

Coating with complete medium

1. In the aliquot containing your rinsed pellet of beads, add 100 µL of complete medium used for the culture of your cells of interest.
2. Vortex the mix for 30 seconds.
3. Before the beads sediment, place the aliquot on a rotating wheel for 3 hours.
4. Conserve the aliquot at 2 to 8°C, with the cap wrapped in Parafilm. If manipulated only in sterile conditions, it can last up to one month.

The resulting concentration expected in the aliquot is 120,000 beads/µL. Accounting for the loss of a fraction of the beads during the rinsing steps, the actual concentration should be around 100,000 beads/µL. To verify one can dilute the solution 100-fold and count the concentration of beads with a typical cell-counting slide.

## Beads Imaging

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| BOX — Guidelines for bead imaging  Before imaging M-450 Dynabeads, the microscope and camera setting have to be adjusted following these principles:   1. The brightness of the light spot below the beads reaches a maximum a few µm below the equatorial plane (around 3.7 µm on our setup for M-450 Dynabeads). We call the position the ‘plane of maximum intensity’. 2. First, focus on a bead in the plane of maximum intensity. Set the exposure time to 5 ms. 3. Set the Köhler illumination conditions on the microscope, then slightly open the field diaphragm to make the light spot sharper. Verify by moving the focal plane up and down. The light spot spreads a bit as you move away from the maximum intensity plane (Fig. 5C). 4. Adjust the light source power to use a significant fraction of the camera dynamic range. Typically, adjusting the maximum intensity to roughly 40’000 grey levels on a 16-bit camera is ideal. The image should never become saturated throughout the light spot. |

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| BOX — Guidelines for optimal 3D tracking of beads  When acquiring a time-lapse, the following points are keys to image the beads to allow a precise 3D tracking:   1. The tracking of the beads in the XY plane will be more precise when the focus is done on the plane where the light spot is the brightest (plane of maximum intensity). 2. The tracking of the beads along the Z-axis will be more precise when the focus is done on a plane located above or slightly below the plane of maximum intensity. This is because the shape of the light spot does not vary much when moving up and down from the plane of maximum intensity, but starts forming specific patterns when one move further below or – even better – above. These patterns facilitate the tracking along Z. 3. As mentioned before, the ideal solution to optimize tracking in both XY and Z is to acquire a small stack of 3 images for each time point. Typically 3 planes 0.5 µm apart in Z and centered on the plane of maximum intensity will allow a very precise 3D localization of the beads. 4. If the conditions of imaging do not allow such Z-stack to be acquired at each time-point, the best plane to localize the bead precisely in XY and Z on a single image is one located slightly above the plane of maximum intensity, where the light spot below the bead starts spreading a little bit: it is the best compromise between points (1) and (2). |

# Magnetic Pincher I – Setup

## Magnetic field generation

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| *# This could be placed here or before, in the presentation of the technique in the results section.*  This technique requires the generation of a uniform magnetic field over the experimental chamber. When exposed to an external magnetic field, superparamagnetic beads will become magnetic dipoles, with a magnetic moment that is a function of the applied field. Because the gradient of the external field is negligible, there is no long-range magnetic force and the beads do not drift. Instead, the dipolar force generated by each bead causes them to attract each other. This leads to a self-organization of the beads present in the experimental chamber, forming pairs or chains aligned with the magnetic field (Fig. 5A, 4B). We insist here on the importance of the field uniformity: too strong a gradient of magnetic field would cause superparamagnetic beads to drift toward the higher field regions. |

In this work, we used mainly electromagnetic coils to generate the field. This solution required designing a custom stage to be mounted on a microscope, but has a crucial advantage: a control in real time of the field magnitude. As a consequence of Ampere’s law, an electric current circulating through a coil of conductive wires generate a magnetic field that curls around the coils. A pair of such coils positioned symmetrically along a common axis, with a distance between the coils of the same order of magnitude than the radius of the coils, are called pseudo-Helmoltz coils. If the same currents circulate through both coils in the same direction, a quasi-uniform magnetic field will be generated in the space between the coils. The magnetic field magnitude in the center of this system is proportional to the intensity of the electric current.

In practice, our two coaxial coils (custom made by SBEA Technologies, France) are completed with a mu metal core (750 spires; length: 40 mm; inner diameter: 46 mm; outer diameter: 86 mm, see Fig. 4A) to increase the generated field. They are powered by a bipolar operational power supply amplifier 6A/36V (Kepco, USA) controlled by the computer through a data acquisition module (National Instruments, USA). The maximum field generated is 55 mT (which correspond to the maximum supplied current, 6 A) with a gradient less than 0.1 mT·mm−1 over the sample.

We describe further in the chapter another solution to generate a uniform magnetic field, which is lighter, easier to build in a lab and to mount on any microscope. It is called the Halbach array [10], and consist in a set of permanent magnets arranged in a specific geometry around the sample. Its main downside is to be restricted to constant fields.

## Imaging system

To monitor the actin cortex thickness in time, a pair of beads pinching the cortex must be tracked in 3D. This is done by acquiring a time-lapse movie of the beads illuminated in bright field, with the focus on the light spot below the beads. To further improve the precision of the bead tracking, a Z-stack of 3 images is acquired at each time-point. A high magnification and a high dynamic range are required to produce images where the beads can be tracked precisely.

In total, in addition to the magnetic field generator, the imaging setup includes the following elements:

1. 100X objective, NA = 1.4.
2. Bright-field light source with a field diaphragm and a condenser diaphragm on the light path.
3. Orca Flash4 camera (Hammamatsu, Japan) with a 16-bits dynamic range.
4. PIFOC focus scanner to control the position of the focal plane along the Z-axis (PIFOC P-721.CDQ, Physik Instrumente, Germany).
5. Image acquisition software which can control both the camera and the Z-axis, and save along with the images a log file containing the precise acquisition time of each image. We developed a Labview program, with a data acquisition module (NI 6343, National Instruments, USA).
6. An environment control chamber to maintain the temperature of the sample to 37°C throughout the experiment, and ideally control the C02 concentration. We use The Box and The Cube (Life Imaging System, Switzerland).

## Inputs & Outputs -> WRITE

# Magnetic Pincher II – Experimental execution

## General comments -> UPDATE

This protocol was optimized for two cell types: primary mouse dendritic cells (DC) and 3T3 fibroblasts (3T3). In the following protocol, some steps will differ according to which cell type they are applied to. This will be clearly stated. We believe that together, these two cases are representative of a large number of cell types. Importantly, they encompass the cases of strongly adherent cells (3T3) and weakly-adherent ones (DC).

## Cell handling

Goal: Have the cell take up M-450 Dynabeads and transfer them to the experimental chamber. This part of the protocol is very dependent on the cell type used. We propose two options: one for the primary mouse Dendritic Cells (DC) that can take beads up fast and are not strongly adherent, and another for the 3T3 fibroblasts that take up beads less efficiently and are strongly adherent.

Incubation with beads (see Note 4)

1. 2 days before the experiment, seed approximately 2 × 105 cells in a 25 cm² culture flask.
2. Mix 6 µL of the coated M-450 Dynabeads solution (see 3.3.3) in 1 mL of warm culture medium. Vortex and add to the flask. Gently rock the flask to homogenize the distribution of beads.
3. After 2 days, a significant fraction of cells are expected to have ingested at least one bead (10 to 50% according to the bead preparation). Verify that hist is the case using a simple phase contrast microscope.

Transfer in experimental chamber (see Note 3)

1. On the day of the experiment, detach the 3T3 cells from their flask with TrypLE and resuspend them in warm imaging medium.
2. In a micropatterned dish with 20 µm fibronectin discs (as prepared in 3.3.4) transfer 2 mL of cell suspension at 1.5× 105 cells/ml. Let settle and adhere to the patterns for 20 min, at 37°C 5% CO2.
3. Check that most micropatterned discs are occupied by a cell that has started to adhere on the substrate.
4. Flush the bottom of the chamber to remove the non-adherent cells. To do this, hold a pipet in each hand and inject warm medium on one side as you aspire on the other side of the chamber. Never let the chamber dry. The flushing will generate a flux of medium in the chamber and efficiently remove excess cells, leaving only the adherent ones.
5. Let the cells adhere completely for 2 hours at 37°C and 5% CO2.

Outer bead addition

1. Prepare a mix of 0.5 mL of warm imaging medium and 2.5 µL of the coated beads solution (see 3.3.3). Vortex and add to the dish.
2. Optional - Only if the microscope incubator does not control CO2. Add 0.5 mL of imaging medium supplemented with HEPES solution to bring the concentration of HEPES in the medium to 20 mM.

## Magnetic Pincher imaging

After placing the sample onto the microscope stage where the magnetic field generator is mounted, the first step is to acquire Z-stacks of beads. They will be used to compute a “Depthograph” (see section 3.4 and Fig. 8). It is preferable to perform this step in the experimental dish containing the cells in the absence of a magnetic field: the formation of chains of beads would hinder the acquisition of clean Z-stacks. Then, the magnetic field can be applied to trigger the beads self-organization in the whole chamber, and many pinching event should occur simultaneously. For each imaged cell, acquire a 10 min time-lapse with a stack of images at different Z-positions for each time point (Fig. 6A). Together with a log file indicating the precise time for the acquisition of each image, these movies are the raw data processed by the image analysis software to determine the cortex thickness as a function of time.

Z-scan of beads (reference Depthograph)

1. Set the microscope light power and the field aperture as explained in 3.2.3. These setting should not change throughout the whole imaging session.
2. Position one or several beads in the field of view. These beads should be clearly separated from each other, perfectly still, and their image not affected by any object in the chamber or on the optical path. Position the focus in the equatorial plane of the beads (see 3.2.3).
3. Acquire a Z-stack of these beads: 401 steps every 20 nm, for a total course of 8 µm. The piezo element must be used to ensure precision of the displacement along Z.
4. Repeat steps 2 and 3 until at least 8 Z-stacks of beads have been successfully acquired.

Magnetic Pincher on live cells (see Notes 5, 6)

1. “Switch on” the magnetic field generator. This is done by either placing the Halbach array on its support, around the sample; or by sending current in the Helmholtz coils. The field magnitude should be between 3 and 10 mT, typically 5 mT, and be kept constant across experiments to obtain comparable results. It is strongly recommended to use a Gaussmeter to measure the applied field in situ.
2. Position a cell whose cortex is pinched by a pair of beads in the field of view. In addition to the guidelines defined in section 3.2.4, the following criteria must be met:

These beads should be as much as possible aligned along the magnetic field lines and in the same plane.

The light spot below them should not be hindered by extra or intra-cellular objects.

The pair of beads can be part of a longer chain that extend into, or out of the cell, or even both. However, beads pinching the cortex must have a maximum of 2 nearest-neighbors (one of them being automatically the other bead of the pair) that are well aligned with the line of magnetic field. Otherwise the magnetic field in the region of the beads will not be properly defined.

1. Acquire a time-lapse of these beads (Fig. 6A, 5B):

2 to 10 minutes, one z-triplet of images every 600 ms, for a total of 200 to 1000 time-points. Shorter durations allow for more movies of different cells to be acquired. Longer time-lapse movies allow for a more extended measurement of each cell cortex behavior.

Each z-triplet is a short Z-stack of 3 images, with a step of 0.5 µm, ideally centered on the plane of maximum intensity. The piezo element must be used to ensure precision of the displacement along Z.

Allow at least a delay of 50 ms between each step of the Z-stack, to ensure the precision of the positioning along Z.

1. Make sure that the software used for the acquisition also saves the precise time at which each image was taken. This can be attached to the images metadata or saved in a separate log file.
2. Repeat steps 2 and 3 to acquire a dataset representative of your cell population. A given chamber should not be images for longer than 2 hours. Typically 10 to 20 cells should be acquired in each chamber, depending on the duration of the acquired time-lapse movies.

# Magnetic Pincher III – Image analysis

This section details the image analysis workflow used to extract the cortical thickness as a function of time from the acquired time-lapse images. It requires two different software packages: ImageJ (in our case Fiji [16]) and Python. To analyze a group of time-lapse imaged from the same dish, the first task is to generate a Depthograph. It simply consists of a YZ-plane averaged profile of the bead, used to determine the relative positions of the beads along the Z-axis [17]. Then a simple tracking algorithm is applied to the time-lapse movies of Magnetic Pincher, to compute the trajectories of the beads in 3D. As many other tracking algorithms, ours proceed in two classic steps: (i) Objects segmentation and (ii) Frame-to-frame matching. Step (i) is done semi-automatically with Fiji while step (ii) has been automatized in a Python function.

The Python code can be found on Github: https://github.com/jvermeil-biophys/CortExplore\_MIMB.git. The main libraries required to to run the code are the following: os, time, numpy, pandas, scipy, scikit-image [18], matplotlib and pyautogui.

|  |
| --- |
| BOX — Bead center detection in Fiji  This detection method is routinely used across many of the image analysis workflows presented in this chapter. While simple, it allows a detection of the center of a bead in the XY plane with a very high precision. It consists in segmenting of the light spot below a bead, then computing its center of mass, using the pixel value as weight (see Fig. 7).   1. In the Fiji software, open an image or a movie containing beads (Fig. 7A). 2. In “Set Measurement” select “Area”, “Standard deviation”, “Center of Mass”, “Stack Position”. 3. Use “Image/Adjust/Threshold” to manually segment the light spots under the beads (Fig. 7B, 6C). Enable the “Stack histogram” option. If the file analyzed is a movie, the same threshold value is used to segment the beads in all the frames of the movie. The regions segmented should cover each light spot, be roughly circular and have a diameter around 1 to 2 µm. Leave the “Threshold” dialog box open with the thresholded regions visible on the image. 4. Use the “Image Particle” tool to measure the properties of each region (Fig. 7D), with the following options enabled: “Display results”, “Exclude on edges”, “Include holes”, “Clear results”. Adjust the area and circularity criterion so that the only objects analyzed are the segmented light spots. Select “Show: Outlines” to visually check that all the light spots were analyzed. 5. Save the Results table (Fig. 7E) in .txt format. |

## 3.4.2 Generate a Depthograph from the bead Z-stacks (see Note 8)

In order to make a reference Depthograph, process bead Z-stacks acquired for a given experiment to compute a typical YZ profile of the beads.

Detection of the beads center, in Fiji.

1. In the Fiji software, open a Z-stack of one or several beads (acquired as specified in 3.3.6). For each bead crop a small rectangular region of interest around the bead and save the resulting Z-stack in .tif format.
2. Open one of these single-bead Z-stack.
3. Use the method detailed in BOX to detect the position of the center of the bead on most frames of the Z-stack. Not managing to detect the light spot on the first and the last frames is normal. The light spot should be successfully segmented typically from frame 100 to frame 300 over a total of 401 in the stack, without any misdetection within this range. Check visually with the “Outlines” that it is the case.
4. Save the Results table in .txt format in the same folder as the corresponding .tif single-bead Z-stack. By convention, the name of this .txt file should be the same as the .tif file, appended with “\_Results.txt”.

Computation of the Depthograph, in Python.

This task is performed using the Main\_DepthoMaker.py script from the CortExplore\_MIMB package. In order to run it follow these steps:

1. Gather all the .tif bead Z-stack files for a given experiment in a folder. Each .tif file must be accompanied with the corresponding “\_Results.txt” file generated in the previous step. The naming convention must be strictly respected.
2. Open the Main\_DepthoMaker.py script in any development environment (e.g. Spyder). Run the line importing the depthoMaker() function from SimpleBeadTracker.py. If it returns an error, check that the working environment is defined as the “CortExplore\_MIMB” folder (where the .py files are).
3. Indicate the paths of the relevant directories by filling the dictionary “dictPaths” according to the instructions in comment.
4. Indicate the value of the relevant parameters by filling the dictionary “dictConstants” according to the instructions in comment.
5. Run the line calling the depthoMaker() function. The resulting Depthograph, along with a “\_Metadata.csv” file will be saved in the specified directory.

The precise instructions to run this code are detailed in the Github repository documentation.

As a reference, here are the outlines of the functioning of the depthoMaker() function.

Open a .tif Z-Stack of a bead. Using the previously computed Results file, on each frame, translate the image so that the center of mass of the bead is exactly in the center of the image. This is done using skimage transform.warp() function with a bi-linear interpolation.

On each frame, take the vertical intensity profile of the bead. This can be done by averaging the 5 central vertical lines of the frame. This intensity profile is a 1D array of pixel value.

Concatenate all these arrays along a second dimension to obtain a 2D array of intensity profiles. The first dimension is Y the second is Z. This constitutes a Depthograph for a given bead.

Repeat the steps 1 to 3 for each stack of beads corresponding to an experiment. Average all the Depthographs, by using the plane of maximum intensity to align them. The resulting 2D array is the average YZ profile of beads imaged to produce the Depthograph of a given experiment.

## 3.4.3 Segment the beads with ImageJ

As a first step to track the beads positions in the Magnetic Pincher time-lapse, segment the light spots of the beads of interest in all the frames in ImageJ / Fiji and determine the position of their center.

1. In the Fiji software, open a Magnetic Pincher time-lapse. If necessary convert it to .tif format.
2. If the images are organized as Z-T hyperstacks, flatten to obtain a 1-dimensionnal stack where images are ordered in the following way: (t1, z1), (t1, z2), (t1, z3), (t2, z1), (t2, z2), (t2, z3), …, (tN, z1), (tN, z2), (tN, z3); where t1 … tN are all the successive time-points and z1, z2, z3 are the lower, middle and upper Z-planes, in that order. To do so, use the function “Image/Hyperstack/Hyperstack to Stack”.
3. Use the method detailed in BOX to detect the position of the center of each bead of interest in the time-lapse. Check visually with the “Outlines” plot that the light spots corresponding to the beads of interest were analyzed in nearly all the frames. Save the Results table in .txt format. By convention, the name of this .txt file should be the same as the .tif file, appended with “\_Results.txt”.
4. Repeat steps 1 to 3 for each of the acquired Magnetic Pincher time-lapse.

## 3.4.4 Track the beads in 3D with a custom-made Python algorithm

The second step of the tracking is performed using the Main\_3DTracker.py script from the CortExplore\_MIMB package.

1. Gather all the .tif Magnetic Pincher time-lapses files for a given experiment in a folder. Each .tif file must be accompanied with 2 other files: (i) the corresponding “\_Results.txt” file generated in the previous step and (ii) a file containing the precise date in millisecond of each image as a single text column. By convention, the name of this .txt file should be the same as the .tif file, appended with “\_Timepoints.txt”.
2. Open the Main\_3DTracker.py script in any development environment (e.g. Spyder). Run the line importing the mainTracker() function from SimpleBeadTracker.py. If it returns an error, check that the working environment is defined as the “CortExplore\_MIMB” folder (where the .py files are).
3. Indicate the paths of the relevant directories by filling the dictionary “dictPaths” according to the instructions in comment.
4. Indicate the value of the relevant parameters by filling the dictionary “dictConstants” according to the instructions in comment.
5. If necessary set optional parameter values by filling the dictionary “dictConstants” according to the instructions in comment.
6. Run the line calling the depthoMaker() function.
7. For each Magnetic Pincher time-lapses file, the program will start by displaying the first frame, where detected beads labeled with orange crosses. If both of the beads pinching the cortex are properly detected on this first image, click on “Yes” then click on the positions of the two beads (the clicks on the image do not have to be very precise). If one of the two beads is not detected, click on “Next Frame” and continue.
8. As the program matches the positions of the beads frame by frame to build their trajectories, it might happen that the algorithm lose track of one of the beads of interest, either because it was not detected on a given frame, or because its frame-to-frame motion was significant. Then, the program displays this frame. Once again, if both of the beads pinching the cortex are properly detected on this first image, click on “Yes” then click on the positions of the two beads (the clicks on the image do not have to be very precise). If one of the two beads is not detected, click on “No”.
9. Once the program have built the trajectories, it will ask the user to assess for each of the beads of interest whether it is in or out of the cell (useful to save the trajectories), and whether is has 1 or 2 neighbors (useful to compute the pinching force).
10. [Automatic step] After these steps building the trajectories of the beads in the XY plane, the program automatically computes the motion of the beads along the Z-axis by comparing for each frame the vertical profile of the beads of interest to the Depthograph generated for this experiment. This step can be time-consuming.
11. [Automatic step] From the XY and Z positions of the beads, the program computes the 3D center-to-center distance between the beads.
12. [Automatic step] The program computes the force applied by the beads on the cortex for each time-point, using the method detailed in [8].
13. [Automatic step] Finally, the program saves a “\_timeseries.csv” file in the specified folder.

The resulting “\_timeseries.csv” file is a table containing the evolution with time (column “T”) of different quantities: the beads center-to-center distances along each axes (“dx”, “dy”, “dz”), in the XY-plane (“D2”) and in 3 dimensions (“D3”). It also contains the magnetic field (“B”, constant) and the force (“F”, varying due to the fluctuations in distance). To compute the time-resolved thickness of the cortex, subtract the beads average diameter (see 3.3.2) to the distance in 3 dimensions (“D3”). Average thickness of the cortex and fluctuation amplitude can be readily obtained from this time-series (see Note 11).

|  |
| --- |
| BOX — Try the code!  \*Instructions to find the code on GitHub and try it on example data!\* |

# Magnetic Pincher with Halbach Arrays

The term “Halbach array” refers to an arrangement of permanent multipole magnets. These arrays can have very diverse properties and application depending on their geometry. Here we use a set of dipolar magnets arranged in a circle (for more details see [11] and specifications in Table 1). It generates a uniform magnetic field inside the circle, and a negligible field outside. The magnets have to be identical in size and magnetization, and arranged so that their direction vector rotates twice faster than their position vector on the circle (Fig. 2A).

In such geometry, the field generated in the center of the Halbach array has the following magnitude:

[Eq. Z]

Where N is the number of magnets used in the array, µ0 is the vacuum magnetic permeability, R is the radius of the circle joining the center of magnets, and m is the magnitude of the magnetic moment of the magnets.

Another useful relationship in Halbach array design is the following: for a given magnet of volume V that contains a uniform field of magnetization M, the resulting magnetic moment m is simply:

[Eq. Z]

As a reference the neodymium N42, of which the cubic magnets we use are made, has a magnetization of MN42 ≈ 1.01 x 106 A/m. Their magnetization is simply deduced from this value and their geometry.

A slightly more complex application of this concept is the nested Halbach array (Fig. 3B). The idea is two use two concentric Halbach array that generate the same field B0 and can be rotated independently. This is possible using larger magnets for the outer array, since as stated by Eq. Z, the generated field magnitude decreases rapidly when the radius of the array increases; this decrease is compensated by increasing the volume of the magnets so they possess a larger magnetic moment (Eq. Z). Therefore, this system can generate uniform magnetic field of any magnitude between ≈ 0 mT (when the two arrays’ fields are in opposite direction) and 2.B0 (when they are in the same direction).

We detail here the specifications of a nested array that we have optimized for this protocol and which is easy to mount on many microscopes, given that it has the outer dimension of a 6 well plate (Table 1, Fig. 3C). The body of the device, meaning the rectangular support and the two rings bearing the magnets, have been 3D-printed (Printer: Fortus 250mc, Stratasys, USA; Material: ABS X-TREME, iSQUARED, Switzerland). The neodymium cubic magnets are commercially available (Supermagnete, Germany). This instance of the nested Halbach array is designed to generate a maximum field of 2.B0 = 8.6 mT.

**Tables**

## **Table 1** – Example of nested Halbach array design – Technical specifications

|  |  |
| --- | --- |
| **Support** | |
| Length x Width x Thickness | 127 x 85 x 3.5 mm |
| **Inner array** | |
| Inner / Central / Outer radius | 21 / 24.75 / 28 mm |
| Height | 5 mm |
| Magnet side length | 3 mm |
| Number, type of magnets | 16, N42 Neodymium |
| **Outer Array** | |
| Inner / Central / Outer radius | 28 / 33 / 38 mm |
| Height | 5 mm |
| Magnet side length | 4 mm |
| Number / type of magnets | 16 / N42 Neodymium |
| **Magnetic Properties** | |
| Maximum magnetic field magnitude  (arrays in the same direction) | Ideally: 8.6 mT  Experimentally: 8.3 mT |
| Minimum magnetic field magnitude  (arrays in opposite directions) | Ideally: 0 mT  Experimentally: 0.2 mT |
| Gradient over the central 1 cm region | < 0.11 mT.mm-1 |
|  |  |

## **Table 2** - Comparison of the two magnetic field generation solution

|  |  |  |
| --- | --- | --- |
|  | Halbach array | Coils |
| Fabrication | * The body can be 3D-printed * Magnets are available at low cost | * Need of a custom manufacturing |
| Size | Can be designed with the size of a 6-wells plate or a 10 cm petri dish. | Each coil is 40 x 86 mm  (length x outer diameter) |
| Mounting on a microscope | Simple, given the flexible design options. | Require a ≈ 140 x 95 mm rectangular hole in the microscope stage. |
| Generated field | With a simple array: one fixed field, from 1 to 90 mT.  With a nested array: tunable field, from 0 to 30 mT. The adjustments cannot be done live during an experiment. | Field adjustable in live during the experiment by tuning the intensity of the current supplied to the coils. The field can go from 0 to 60 mT, but high magnitudes cannot be maintained too long, due to the Joule effect heating the coils. |

# Magnetic Pincher with Nano-Indenter

## Modifications of the setup

## Modifications of the execution